The Journal of Experimental Biology 207, 1915-1924
Published by The Company of Biologists 2004
doi:10.1242/jeb.00975

Force development, energy state and ATP production of cardiac muscle from turtles and trout during normoxia and severe hypoxia

Johannes Overgaard and Hans Gesser*

Department of Zoophysiology, Institute of Biological Sciences, University of Aarhus, DK 8000 Aarhus, Denmark

*Author for correspondence (e-mail: hans.gesser@biology.au.dk)

Accepted 10 March 2004

Summary

The effects of hypoxia on energy economy of cardiac muscle were compared between the hypoxia-tolerant freshwater turtle at 20°C and the hypoxia-sensitive rainbow trout at 15°C. Isolated ventricular preparations were left either at rest or stimulated at 30 min^{-1} to develop isometric twitch force. Under oxygenated conditions, twitch force and oxygen consumption were similar for the two species. Overall metabolism was reduced during severe hypoxia in both resting and stimulated preparations and under these conditions most of the ATP production was derived from anaerobic metabolism. During hypoxia, a metabolic depression of approximately 2/3 occurred for non-contractile processes in both turtle and trout preparations. During hypoxia, lactate production and residual oxygen consumption were similar in turtle and trout. Cellular energy state and phosphorylation potential decreased during severe hypoxia in both species and this reduction was more severe in preparations stimulated to contraction. However, in turtle ventricular preparations the energy state and phosphorylation potential stabilised at higher levels than in trout, and turtle preparations also maintained a higher twitch force throughout the hypoxic period. Moreover, twitch force relative to total ATP hydrolysis was markedly increased during hypoxia in turtle while this ratio was unchanged for trout. The main findings of this study are: (1) cellular energy liberation and the energy demand of non-contractile processes decreased to similar levels in hypoxic turtle and trout myocardium; (2) turtle myocardium maintained a substantially higher cellular energy state and twitch force development than trout myocardium during hypoxia and (3) the ratio of twitch force to ATP hydrolysis increased during hypoxia in turtle but was unchanged in trout. It is possible that this superior economy of the contracting turtle myocardium contributes to the remarkable hypoxia tolerance of freshwater turtles.

Key words: oxygen consumption, lactate production, cellular energy state, phosphorylation potential, cost of contraction, twitch force, metabolic depression, *Trachemys scripta*, *Oncorhynchus mykiss*.

Introduction

Freshwater turtles possess high hypoxia tolerance relating to their diving behaviour and ability to hibernate for several months without access to air (Jackson, 2000, 2002). While sustained cardiac function during hypoxia is essential for survival (Belkin, 1968), cardiac performance is markedly reduced and may depend on the high circulating levels of catecholamines during anoxia (White and Ross, 1966; Jackson, 1987; Keiver et al., 1992; Wasser and Jackson, 1991; Hicks and Wang, 1998).

The ability of cardiac muscle from turtles to tolerate anoxia is associated with a superior maintenance of cardiac cellular energy state relative to most other species of ectothermic vertebrates (Wasser et al., 1990; Jackson et al., 1995; Hartmund and Gesser, 1996). Furthermore, it seems that the myocardium preserves most contractile activity during severe hypoxia/anoxia at low workload (Wasser et al., 1990; Jackson et al., 1995; Hartmund and Gesser, 1996). This ability has been related to a high glycolytic capacity (Bing et al., 1972) and is reflected in a high anaerobic metabolic capacity relative to aerobic capacity (Christensen et al., 1994). Other studies, however, indicate that neither the glycolytic nor the mechanical capacities of turtle hearts are exceptional, and it has been suggested that their anoxia tolerance relates primarily to downregulation of metabolic requirements so that cellular energy state is protected (Arthur et al., 1997; Farrell et al., 1994).

Studies of perfused hearts from turtle and trout indicate that work output is achieved at similar rates of ATP consumption during full oxygenation and severe hypoxia (Reeves, 1963b; Arthur et al., 1992, 1997). However, recent studies on rats, guinea pigs and frogs raised the possibility that the energetic cost of developing tension during hypoxia is lower in hypoxia-tolerant vertebrates (Joseph et al., 2000). In addition to inherent differences in the efficiency of the contractile system, a low cost of contraction (i.e. overall cellular ATP consumption/force developed) would also occur if the metabolic demands

of non-contractile processes are suppressed. This seems to be the case during anoxia in rabbit papillary muscle, where cellular ATP production seemed insufficient to cover energy-demanding processes other than contractility (Mast and Elzinga, 1990). As metabolism of whole animals and tissue culture of turtles is markedly reduced during anoxia (Jackson, 1968; Hochachka et al., 1996; Lutz and Nilsson, 1997), it is an interesting possibility that the apparent cost of cardiac contraction is also reduced, either as a result of changes in the economy of the contractile system or through a reduced cost of non-contractile processes.

Here, we investigate the relationships between cellular energy production, force development and cost of non-contractile processes in ventricular muscle strips from the turtle *Trachemys scripta*. Our preparation allowed for simultaneous measurements of metabolism and force production in resting and paced ventricular strips. Mechanical performance was assessed as isometric twitch force, and cellular ATP production was derived from recordings of oxygen consumption, lactate production and concentrations of cellular high-energy phosphates. The preparations were exposed to adrenaline during hypoxic recordings because substantial increases in plasma catecholamines have been observed in both turtle and trout subjected to hypoxia/anoxia (Wasser and Jackson, 1991; Keiver et al., 1992; Reid and Perry, 2003).

The results from the hypoxia-tolerant turtle were evaluated relative to a parallel series of experiments performed on cardiac muscle from more hypoxia-sensitive rainbow trout (*Oncorhynchus mykiss*). Relative to the turtle heart, the trout heart has an inferior ability to maintain force development and cellular energy state during hypoxia (Arthur et al., 1992; Hartmund and Gesser, 1996).

Materials and methods

Experimental animals

Freshwater turtles (*Trachemys scripta* Gray; mass, 262±40 g) were obtained from Lemberger (Oshkosh, WI, USA). They were housed at 20°C in tanks with free access to basking platforms at a 12 h/12 h light:dark cycle and fed 2–3 times a week with lettuce, mussels and fish meat. Rainbow trout (*Oncorhynchus mykiss* Walbaum; mass, 361±20 g) were obtained from local breeders, fed daily with commercial fish food (Ecolife 19; Biomar, Brande, Denmark) and maintained in tanks with recirculating well-aerated freshwater at 12–15°C, a temperature range covering their breeding temperature.

Myocardial preparations

Animals were decapitated, and the hearts were rapidly excised and transferred to an ice-cold physiological solution containing (in mmol l^{-1}) 125 NaCl, 2.5 KCl, 0.94 $MgSO_4$, 1 NaH_2PO_4, 1.2 $CaCl_2$, 10 glucose and 30 and 15 $NaHCO_3$ for turtle and trout, respectively.

From each ventricle, 1 mm-thick ring-shaped preparations were obtained by transverse cuts. The preparations were weighed, mounted on two hooks and immersed in physiological solution continuously gassed with a mixture of 88% O_2, 2% CO_2 and 10% N_2 for turtle and 89% O_2, 1% CO_2 and 10% N_2 for trout. pH of the physiological solution was ~7.7 in both the turtle experiments at 20°C and in the trout experiments at 15°C. This pH value accords reasonably with *in vivo* recordings from freshwater turtles and trout at the same temperatures (Nicol et al., 1983; Julio et al., 1998).

Muscular contractions were elicited by a Grass SD 9 stimulator (Quincy, MA, USA), which delivered electrical square pulses of 5 ms and a voltage 50% above that eliciting maximal twitch force. The preparation was stretched with a micrometer screw to produce ~90% of maximal twitch force, as this would not overstretch the preparations while leaving almost all myosin heads active.

Experimental setup

The experimental protocol was designed to evaluate the effects of severe hypoxia on the energetic state, ATP production and its relation to force production in ventricular preparations from turtle and trout in two experimental series. In series 1, intracellular concentrations of high-energy phosphates and lactate were measured. In series 2, ATP production was calculated from the oxygen consumption during oxygenation and from both oxygen consumption and lactate production during the period of severe hypoxia.

Series 1

This series was designed to measure tissue content of ATP, ADP, AMP, phosphocreatine (PCr), creatine (Cr) and lactate together with twitch force. Six ring-shaped preparations were mounted in parallel setups as open ellipsoids around two hooks and immersed in 50 ml physiological solution in thermostatted baths. The upper hook consisted of a thin glass rod connected to a force transducer (Fort 10; World Precision Instruments, UK), while one of two platinum electrodes used for pacing was used as the lower hook.

Series 2

The experimental setup for simultaneous measurement of twitch force, oxygen consumption and lactate production was similar to that described by Kalinin and Gesser (2002). Briefly, ventricular rings were mounted around two hooks of stainless steel in a tube-formed glass chamber (diameter of 12 mm and a volume of 2.56 ml) that could be sealed with a glass stopper. The lower end of the ring-shaped preparation was mounted on a stainless steel hook and the upper end was mounted on a stainless steel rod attached to a force transducer through a 1 mm hole in the stopper. Electrical stimulations were obtained from two chloridized silver electrodes situated on opposite sides of the preparation and connected to the stimulator through the stopper. To avoid polarization, the polarity of the electrodes was alternated between pulses. A peristaltic pump circulated physiological solution at a rate of 3 ml min^{-1} between the chamber and a reservoir through two stainless steel tubes penetrating the stopper. The reservoir of 15 ml was continuously gassed with the desired gas mixture. Oxygen

tension in the chamber was recorded with an oxygen electrode (Radiometer, E5046) placed horizontally so the tip was just inside the chamber. The chamber, the oxygen electrode and the reservoir were thermostatted to the desired temperature and the chamber solution was continuously stirred using a glass-covered magnetic stir bar.

Signals from the force transducers and oxygen electrode were recorded with a Biopac MP100 data acquisition system (Biopac Systems, Inc., Goleta, CA, USA) at 50 Hz. At the experimental stimulation rate (30 min^{-1}) the twitch duration was approximately 2 s and 1 s for the turtle and trout preparation, respectively.

Experimental protocol

The experimental protocol and time course of series 1 and 2 were chosen to obtain measurement of twitch force, energy production and energy state after stabilisation under oxygenation and severe hypoxia, respectively.

Series 1

When the ventricular rings had been mounted, they were stretched to produce 90% of maximal twitch force and allowed 30 min for twitch force to stabilise. Then, stimulation was switched off for three preparations while stimulation was continued at 30 min^{-1} for three other preparations. Resting heart rates of turtle and trout have been reported to be ~25 min^{-1} for turtles and rainbow trout at 22°C and 15°C, respectively (Hicks and Farrell, 2000; Altimiras and Larsen, 2000). In the present study, a rate of 30 min^{-1} was chosen to obtain clearly increased energy consumption in the force-developing preparations relative to that of the resting preparations. After 30 min at high oxygenation, one contracting and one resting preparation were clamped with aluminium pliers, pre-cooled in liquid nitrogen and the samples were stored at –80°C. Immediately thereafter, stimulation frequency was reduced to 12 min^{-1} and nitrogen was exchanged for oxygen, so that the physiological solution in all baths was made severely hypoxic. After 30 min of severe hypoxia, 10 μmol l^{-1} adrenaline was added to the baths. While this concentration of adrenaline is likely to be well above the *in vivo* range for both species, it gives a well-defined and close to maximal adrenergic stimulation of twitch force (Nielsen and Gesser, 2001). 10 min after addition of adrenaline, one contracting and one resting preparation were sampled and stimulation frequency of the one remaining contracting preparation was elevated to 30 min^{-1}. After an additional 30 min period of continued severe hypoxia, the two last preparations were sampled (Fig. 1).

Series 2

In the experiments for determination of oxygen consumption, lactate production and twitch force, the preparations were run as in series 1 with respect to stimulation frequency, stretch, adrenaline exposure and gas mixtures (Fig. 1). Here, every other preparation was stimulated to contraction or left at rest, respectively. Following the initial stabilization of twitch force, recirculation of chamber solution was stopped for 30 min during which oxygen consumption was measured from the gradual decline in oxygen tension. Then circulation was resumed with the hypoxic solution, which resulted in a chamber oxygen partial pressure (P_{O_2}) of ~1.6 kPa within 10–15 min. After 30 min, 10 μmol l^{-1} adrenaline was added to the reservoir and, 10 min later, circulation was stopped again for 30 min to allow for recordings of oxygen consumption and lactate accumulation during hypoxia (Fig. 1). The reported values of respiration have been corrected for background changes in oxygen tension, which were recorded in the absence of tissue in the chamber, with stimulation either set at 30 min^{-1} or turned off. During full oxygenation, the decrease in oxygen tension in the presence of preparation had to be corrected by maximally 10% and during severe hypoxia by maximally 27%.

Lactate production was determined from the increase in lactate in the tissue and in the chamber solution over the 30 min of hypoxia. Tissue lactate content was assessed on the basis of the values recorded from the samples in series 1, while the increase in chamber solution lactate provided the lactate released from the preparation (Fig. 1). Tissue and solution samples were stored at –80°C until determination of lactate.

Mechanical activity

In the present experiments, mechanical activity was recorded in terms of isometric twitch force, which was used as an indicator of heart function. Thus, a previous study on turtle cardiac muscle showed that the relative effect of anoxia on isometric twitch force and power output was almost identical in preparations that were allowed to shorten after attaining 85% of time to peak force (Shi and Jackson, 1997). The ring preparation was considered suitable for recordings of energy state, energy production and twitch force. Its form, however, makes it less suited for refined recordings of contractile performance. We aimed at having the thickness and cross-section area of the preparations as constant as possible so that twitch force given in mN could be assumed to be proportional to twitch force related to cross-section area.

As an alternative, a time-tension integral has previously been used (Mast and Elzinga, 1990). We found, however, a significant correlation between the effects of severe hypoxia on twitch force and the time-tension integral for both turtle and trout preparations (data not shown).

Biochemical measurements

Myocardial high-energy phosphates were measured using HPLC (Bøtker et al., 1994). Briefly, a 30–60 mg piece of ventricle was homogenised in 1.6 ml of 0.42 mol l^{-1} perchloric acid in a glass-to-glass homogeniser. The homogenate was then centrifuged for 10 min at 3400 ***g***, and the supernatant was separated into two 200 μl samples. The sample used for measurement of creatine compounds (phosphocreatine and creatine) was neutralised with 100 μl KOH (1 mol l^{-1}), and the sample used for subsequent measurement of adenylates (ATP, ADP and AMP) was neutralised with 100 μl of $KHCO_3$

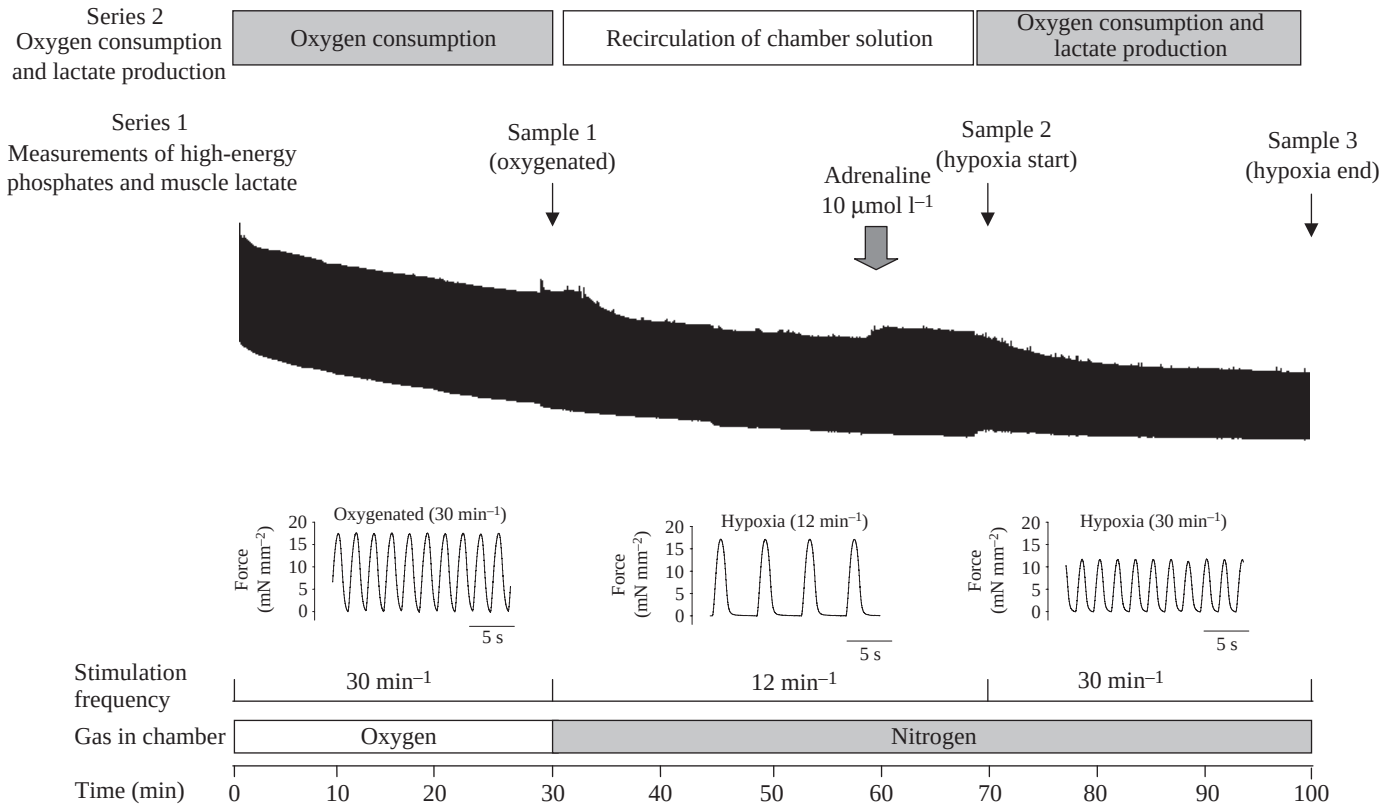

Fig. 1. Experimental steps and time course in series 1 and 2 for ventricular muscle isolated from turtle and trout. Preparations were either left to rest or electrically paced to isometric twitch force development at the rate indicated. A representative recording of twitch force development of a turtle preparation is included. In series 1, preparations were sampled for biochemical determinations at the times indicated. In series 2, twitch force, oxygen consumption and hypoxic lactate production were recorded during 30 min of oxygenation and then during 30 min of severe hypoxia.

(2 mol l^{-1}) and Tris (0.1 mol l^{-1}). After neutralization, both samples were kept on ice for 10 min to ensure precipitation of perchlorate. The neutralized portions were then centrifuged for 5 min (3400 ***g***) and the supernatant stored at –80°C until further analysis. Creatine compounds and adenylate compounds were separated using HPLC (Waters; Minneapolis, MN, USA) with a 10 cm Crompack C18 microsphere-column of 3 μm particle size (Varian, Palo Alto, CA, USA). Creatine compounds were measured at a wavelength of 210 nm using a mobile phase of aqueous buffer containing 0.02 mol l^{-1} KH_2PO_4 and 2.3 mmol l^{-1} tetrabutyl ammonium hydrogen sulphate (TBAHS) run at 1.5 ml min^{-1}. Adenosine nucleotides were measured at a wavelength of 254 nm using a mobile phase of 25% methanol and 75% aqueous buffer containing 0.06 mol l^{-1} KH_2PO_4 and 0.011 mol l^{-1} TBAHS run at 1 ml min^{-1}.

Muscle lactate concentration was measured from the same homogenates that were used for measurements of adenosine products. Lactate concentration of homogenates, reservoir solution (initial) and chamber (final) were determined by transforming it quantitatively along the lactate–dehydrogenase and pyruvate–amino acid transaminase catalysed reactions and recording the amount of NADH formed spectrophotometrically (Lowry and Passonneau, 1972).

Energy state

Gibb's free energy associated with hydrolysis of ATP (ΔG_{ATP}) varies with the phosphorylation potential [ATP/(ADP×P_i)], and Meyer (1988) argued that phosphorylation potential could be estimated by the ratio of PCr/Cr^2, where PCr and Cr represent tissue concentrations of phosphocreatine and creatine, respectively. As hydrogen ions participate in the creatine kinase reaction, changes in ΔG_{ATP} may be confounded by changes in intracellular pH, but this problem seems to be of little importance in the type of preparations used in the present study (Hartmund and Gesser, 1996).

ATP production

Based on previous studies (Arthur et al., 1997; Hansen et al., 2002), ATP production was assumed to be exclusively aerobic during full oxygenation, whereas both lactate production and oxygen consumption were considered during severe hypoxia. In our calculation of ATP production, we assumed that 1 mole of O_2 results in the formation of 6 moles of ATP (Reeves, 1963b; Ferguson, 1987; Mast and Elzinga, 1990) and that 1 mole of lactate equals 1.5 moles of ATP. The lactate/ATP conversion factor is based on the assumption that lactate is derived from glycogen, which seems to be the case for hearts

Table 1. *ATP, ADP, total adenylates (Ad_{tot}), phosphocreatine (PCr), creatine (Cr), total creatine (Cr_{tot}), sum of high-energy phosphates (HEP_{tot}=ATP+0.5×ADP+PCr), phosphorylation potential (PCr/Cr^2) and lactate in cardiac tissue from turtle and trout under oxygenation and after 40 and 70 min of severe hypoxia*

O_2/N_2	Rest/ stim.	ATP (μmol l^{-1} g^{-1})	ADP (μmol l^{-1} g^{-1})	PCr (μmol l^{-1} g^{-1})	Cr	Ad_{tot} (μmol l^{-1} g^{-1})	Cr_{tot} (μmol l^{-1} g^{-1})	HEP_{tot} (μmol l^{-1} g^{-1})	PCr/Cr^2 (μmol l^{-1} g^{-1})	Muscle lactate (μmol l^{-1} g^{-1})
Turtle										
Oxygenated	Rest	2.51±0.32	0.51±0.02	**4.83±0.31**[a]	3.31±0.26[a]	3.18±0.34	**8.14±0.52**	**7.60±0.60**[a]	0.47±0.06[a]	**1.15±0.21**[a]
Hypoxia 40 min	Rest	2.35±0.28	**0.55±0.03**	3.51±0.33[b]	**4.39±0.38**[a,b,c]	3.04±0.30	**7.90±0.63**	6.14±0.59[a,b]	**0.19±0.02**[b]	5.40±1.17[b]
Hypoxia 70 min	Rest	1.88±0.27	**0.50±0.03**	3.38±0.69[b]	**4.04±0.45**[a,b]	2.50±0.31	7.42±1.13	5.51±0.96[b,c]	**0.20±0.01**[b]	**3.48±0.31**[b,c]
Oxygenated	Stim.	2.39±0.26	0.53±0.07	**3.96±0.35**[b]	3.73±0.29[a]	3.08±0.34	**7.68±0.58**	**6.62±0.58**[a,b]	0.30±0.04[c]	**3.29±0.34**[a,c]
Hypoxia 40 min	Stim.	1.74±0.14	**0.52±0.04**	1.78±0.21[c]	**5.17±0.38**[b,c]	2.37±0.17	6.96±0.48	**3.78±0.21**[c]	**0.07±0.02**[d]	**11.31±0.62**[d]
Hypoxia 70 min	Stim.	1.65±0.16	**0.52±0.02**	2.93±0.98[d]	**5.40±0.71**[c]	2.28±0.17	8.33±1.54	4.84±1.03[b,c]	**0.10±0.02**[b,d]	12.85±1.00[d]
Trout										
Oxygenated	Rest	2.56±0.21[a]	0.59±0.05[a]	**7.97±0.87**[a]	4.04±0.49[a]	3.38±0.27[a]	**12.0±1.01**[a,c]	**10.83±1.04**[a]	0.70±0.15[a]	**5.08±0.70**[a]
Hypoxia 40 min	Rest	2.21±0.21[a]	**0.98±0.07**[b]	2.60±0.48[b]	**7.71±0.49**[b]	3.47±0.26[a]	**10.3±0.86**[a,b,c]	5.31±0.70[b,c]	**0.04±0.01**[b]	8.83±1.06[b]
Hypoxia 70 min	Rest	2.38±0.29[a]	**0.84±0.09**[b,c]	3.00±0.39[b]	**7.42±0.76**[b]	3.46±0.39[a]	10.42±0.97[a,b,c]	5.81±0.67[b]	**0.06±0.01**[b]	**6.91±1.06**[a,b]
Oxygenated	Stim.	2.63±0.14[a]	0.68±0.04[a,c]	**8.10±0.61**[a]	4.82±0.41[a]	3.55±0.17[a]	**12.9±0.80**[a]	**11.06±0.74**[a]	0.42±0.07[c]	**6.67±0.64**[a,b]
Hypoxia 40 min	Stim.	1.30±0.24[b]	**0.76±0.06**[c]	0.96±0.18[b]	**7.59±0.49**[b]	2.32±0.31[b]	8.54±0.61[b]	**2.64±0.44**[c]	**0.02±0.003**[b]	**16.52±1.33**[c]
Hypoxia 70 min	Stim.	1.46±0.21[b]	**0.79±0.04**[c]	0.91±0.16[b]	**8.69±0.49**[b]	2.56±0.22[b]	9.60±0.47[c]	2.77±0.36[c]	**0.01±0.003**[b]	15.62±3.13[c]

Values are means ± S.E.M. (N=6 for all groups except oxygenated trout preparations for which N=11/12). Dissimilar letters indicate significant differences due to treatments for turtle and trout, respectively, and differences between turtle and trout are indicated by presenting the values in bold.

from turtle at low workloads (Reeves, 1963a). ATP production was related to twitch force (reported in mN).

Statistics

Differences in high-energy phosphates or muscle lactate due to treatment were examined using a one-way analysis of variance (ANOVA), where a *post hoc* Fisher LSD test was employed to identify significant differences. If data did not fulfil the requirement of normal distribution, a one-way ANOVA on ranks was employed using an SNK *post hoc* test to identify differences. ANOVAs were run separately for turtle and trout, and species-specific differences were examined using Student's *t*-test. Student's *t*-test was also applied to identify differences in ATP production, twitch force and the relation between these two parameters. In all cases, *P*-values below 0.05 were regarded as significant and all results are presented as means ± S.E.M.

Results

Tissue energy state, lactate and twitch force

Table 1 presents concentrations of high-energy phosphates and lactate in myocardial preparations from turtle and trout obtained from experimental series 1. In this series, twitch force for turtle cardiac muscle was reduced to 54±3% and 51±3% of the oxygenated levels after 40 and 70 min of severe hypoxia, respectively. The corresponding values for trout were 35±6% and 25±8%. At both times, the reduction of force was significantly larger for trout than for turtle.

Turtle

As seen in Table 1, neither stimulation nor hypoxia affected myocardial concentrations of ATP, ADP or total adenylates (Ad_{tot}). During oxygenated and hypoxic conditions, stimulation caused a decline in PCr and the phosphorylation potential (PCr/Cr^2). Hypoxia resulted in decreases in PCr, PCr/Cr^2 and in the sum of high-energy phosphates (HEP_{tot}), and in stimulated hypoxic preparations this resulted in increased levels of Cr. During hypoxia, PCr and PCr/Cr^2 decreased to a new stable level in both resting and stimulated preparations. As a result, the energy state of the preparations did not change significantly between 40 and 70 min of hypoxia. Muscle lactate levels increased under hypoxia and reached significantly higher levels in the stimulated than in the resting preparations. Irrespective of stimulation, the increases in muscle lactate levelled off with no significant change between 40 and 70 min of hypoxia (Table 1).

Trout

In oxygenated trout preparations, stimulation reduced phosphorylation potential without significantly affecting other parameters (Table 1). During hypoxia, ATP and Ad_{tot} levels were stable in resting preparations even though ADP increased. PCr fell to 35% and HEP_{tot} to 50% of the levels during oxygenated conditions, and the decrease in PCr was attended by an equimolar increase in Cr. The reduction in energy state during hypoxia was exacerbated by stimulation, as ATP and Ad_{tot} fell while ADP increased. Furthermore, PCr fell to 12% of the oxygenated value and, although Cr increased, there was

a decrease in Cr_{tot}. These changes were associated with a marked reduction in phosphorylation potential and HEP_{tot}, which fell to 25% of initial values. Energy state in both resting and stimulated preparations attained a new stable level after 40 min with no changes occurring between 40 and 70 min of severe hypoxia.

Myocardial lactate concentration of resting preparations only increased slightly during hypoxia. Stimulation did not affect muscle lactate concentration under oxygenation but led to an approximate doubling of muscle lactate in hypoxia. Muscle lactate stabilized with no significant change between 40 and 70 min of hypoxia.

Turtle vs *trout*

Cr_{tot}, PCr and HEP_{tot} were higher for trout than for turtle during oxygenated conditions. However, PCr and HEP_{tot} fell to the same level as in turtle during hypoxia in resting preparations and became even lower than in turtle in stimulated hypoxic preparations. The larger changes in these parameters for trout were accompanied by significantly lower phosphorylation potentials of the hypoxic trout preparation at both rest and stimulation (Table 1).

The changes in muscle lactate due to stimulation and hypoxia were qualitatively similar for both species, although trout had significantly higher muscle lactate concentrations during oxygenation.

Oxygen consumption, lactate production and force

Stimulation at 30 min^{-1} led to a 2–3-fold increase in oxygen consumption in both species. During severe hypoxia, anaerobic ATP production was dominant, and oxygen consumption was reduced to less than 10% of the aerobic level in both resting and contracting preparations from both species (Fig. 2A). Stimulation increased lactate production during severe hypoxia in both species (Fig. 2B). In both turtle and trout, hypoxia reduced ATP turnover to ~30–35% of the values during oxygenation for resting preparations and to ~25% for stimulated preparations (Fig. 2C). There were no significant differences between turtle and trout in either oxygen consumption or lactate production in any of the experimental situations so overall ATP production was similar in both species (Fig. 2C). It should be noted that lactate production was calculated exclusively on the basis of the change in chamber solution, as tissue lactate was unchanged during the recording period (Table 1).

When compared with ATP production, twitch force is represented by the average obtained over the 30 min when oxygen consumption and lactate production were recorded (Fig. 1). All recordings during hypoxia were performed in the presence of 10 μmol l^{-1} adrenaline that had been added 10 min before the start of the 30 min period of recording. Within these 10 min, hypoxic twitch force increased by 31±9% and 104±26% for turtle and trout, respectively. Subsequently, twitch levelled off and then progressively decreased during the following 30 min of hypoxia (Fig. 1). As preparation thickness and cross-section area were kept as constant as possible, the force values recorded were assumed to be proportional to force related to cross-section area. This assumption accords with the finding that twitch force in the present study (Fig. 2) and twitch force related to the estimated cross-section area (mN mm^{-2}) in a previous study on identical ring preparations under the same control conditions (Kalinin and Gesser, 2002) showed a similar insignificant tendency to be higher for trout than for turtle.

In turtle, hypoxia reduced average twitch force by one-third, although this was not significant. In trout, average twitch force decreased significantly by two-thirds under hypoxia, and the change in twitch force due to hypoxia was significantly larger for trout than for turtle (Fig. 2D). The changes in twitch force during hypoxia in series 2 did not differ significantly from those recorded in series 1. During oxygenation, ATP production, twitch force and twitch force/ATP production ratio were similar in both species. However, during hypoxia, twitch force decreased less relative to ATP production in turtle and, consequently, the economy of force development appeared to increase as the twitch force/ATP production ratio increased more than twofold (Fig. 2E). In trout, the changes in ATP production and twitch force were of similar magnitude, leaving the twitch force/ATP production ratio, and thus the apparent cost of contraction, unchanged.

Discussion

Preparation

Oxygen consumption of the ventricular rings of both turtle and trout was similar to previous studies on cardiac preparations from frogs, rainbow trout and freshwater turtles (Syme, 1994; Harwood et al., 2002; Kalinin and Gesser, 2002) but lower than that of perfused hearts working at low workload (Arthur et al., 1992, 1997). Some of these differences may relate to differences in mechanical activity. Thus, due to the Fenn effect, energy consumption increases in preparations, such as the perfused heart, where the myocytes shorten. However, even the estimated oxygen consumption of resting perfused hearts (Arthur et al., 1997) was approximately twice that recorded in the present study. Hence, metabolic turnover in our preparations seems, for somewhat unclear reasons, to be lower than that of perfused hearts. Our preparations may, however, compare well with the very low energy turnover of the anoxic turtles (Hicks and Farrell, 2000).

Energy metabolites and twitch force

During severe hypoxia, cardiac ATP levels were generally well defended and only decreased significantly in stimulated hypoxic trout preparations. There was, however, a reduction in the energy state (estimated from PCr/Cr^2), as observed previously in cardiac strips and *in situ* perfused hearts (Wasser et al., 1990; Arthur et al., 1992; Jackson et al., 1995; Hartmund and Gesser, 1996). The reduction in energetic state during hypoxia was considerably smaller in turtle than in trout but, for both species, energy state stabilised at a higher level during rest than during stimulation. The larger reduction in cellular energy state of trout not only indicates a greater drop in ΔG_{ATP}

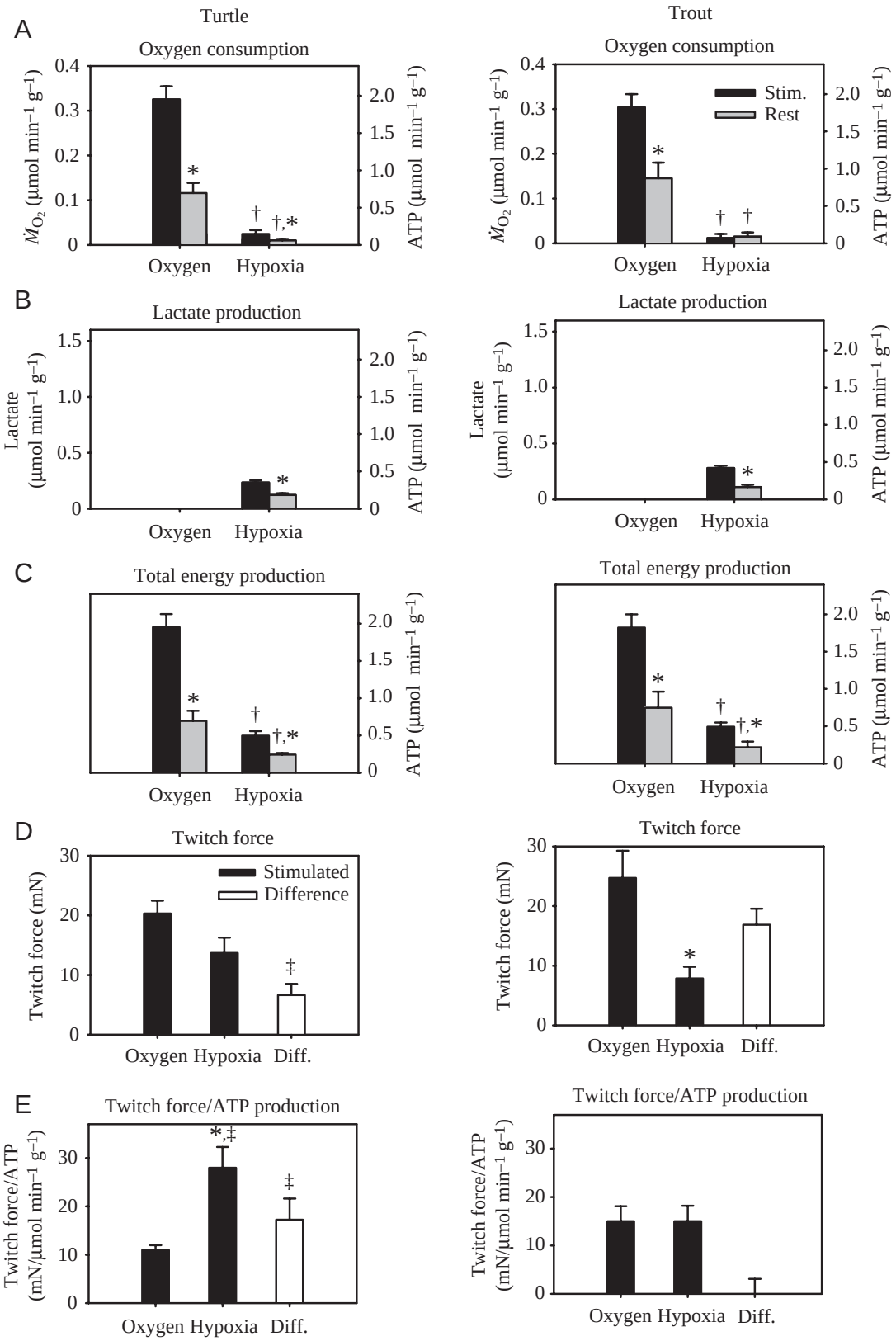

Fig. 2. Oxygen consumption ($\dot{M}_{O_2}$) (A), lactate production (B), total ATP production (C), twitch force (D) and twitch force relative to total ATP production (E) are presented for isolated turtle (left) and trout (right) ventricular muscle. Preparations were either stimulated at 30 min^{-1} (N=8 for both turtle and trout) or left to rest (N=7 for both turtle and trout). For all parameters, values are means ± S.E.M. of the average obtained over the 30 min period of oxygenation and over the last 30 min of the 70 min hypoxic period, respectively. Asterisks signify differences between stimulation and rest, † signifies differences between oxygenation and hypoxia and ‡ signifies differences between turtle and trout.

(free energy liberation of ATP hydrolysis) but also a greater increase in the cellular concentration of inorganic phosphates, $[P_i]$. Furthermore, trout cardiac muscle has a higher concentration of creatine compounds than turtle (Christensen et al., 1994; Hartmund and Gesser, 1996) that allows for larger increases in $[P_i]$, which may explain the larger reduction of twitch force during hypoxia in trout compared with turtle. Increased $[P_i]$ has, together with intracellular acidosis, been suggested as the main cause of contractile failure when cellular energy production is compromised (e.g. Allen et al., 1985; Godt and Nosek, 1989; Arthur et al., 1992; Hartmund and Gesser, 1996), but this effect may vary among species and may depend on energy state (Jensen and Gesser, 1999).

Cellular energy production and twitch force

After 40 min of severe hypoxia, ventricular ATP production had decreased to similar rates in the two species, but the stabilisation occurred at a higher cellular energy state in the turtle. Hence, a balance seemed to be attained at which a similar rate of energy production supported a higher energy state in turtle than in trout cardiac muscle. While it is unclear how this difference between turtle and trout cardiac muscle is achieved, overall ATP consumption during the initial period of anoxia decreases faster in turtle than in trout (Hartmund and Gesser, 1996). The ability of the turtle heart to balance ATP production and consumption during hypoxia with small reductions in energetic state may be due to their superior ability to downregulate metabolic requirements (e.g. Lutz and Nilsson, 1997; Jackson, 2000, 2002). Indeed, metabolic rate of anoxic whole turtles, turtle brain slices and turtle hepatocytes is reduced to 10–40% of normoxic levels (Jackson, 1968; Hochachka et al., 1996; Lutz and Nilsson, 1997). A metabolic depression resembling that found in other tissue also seems to occur in resting turtle cardiac muscle, where the ATP turnover fell to 35% of oxygenated values. However, hypoxic metabolic depression is, at least regarding cardiac muscle, not unique for turtle as we recorded similar reductions in resting metabolic rate of hypoxic trout preparations.

While ATP production in hypoxic stimulated preparations fell by the same proportions in turtle and trout, twitch force decreased more in trout. Consequently, cardiac force development relative to ATP production was markedly increased during hypoxia for turtles while essentially unchanged in trout. The cost of contraction is estimated on the basis of the total rate of ATP production and the force developed. Suppression of energy-demanding processes not related to contraction would, however, enlarge the fraction of ATP production available for force development. Thus, given the decreased ATP turnover at rest, the cost of contraction should appear to decrease. The results from turtle myocardium accord qualitatively with this, although the increase in the force/ATP production ratio seems too large to be explained exclusively by suppression of non-contractile processes. By contrast, the force to ATP ratio was unchanged in trout despite a considerable decrease in ATP consumption of resting preparations. It seems, therefore, that costs of contractile processes increase in trout. In the estimate of an apparent cost of contraction, overall ATP production of contracting preparations was used directly. It may seem more appropriate to use the ATP consumption remaining after subtraction of that in resting preparations. However, considering the interdependence of different cellular processes, the assumption that ATP production due to contractility and other processes are additive is, in all likelihood, a crude over-simplification. For instance, a substantial part of the oxygen consumption in resting cardiac cells probably supports a proton leak (Mortensen and Gesser, 1999), which is transferred to ATP production in contracting cells (Rolfe and Brand, 1997).

In accordance with our results on turtles, Mast and Elzinga (1990) reported an increased ratio between force and overall ATP consumption in anoxic rabbit papillary muscle due to an apparently complete suppression of non-contractile processes. The increased force to ATP consumption ratio in turtle is, however, not fully explained by suppressed non-contractile processes. Alternatively, a decrease in the apparent cost of muscle contraction may reside in the contractile system itself. It was recently reported that the cost of force development increased under hypoxia in isolated hypoxia-sensitive cardiac muscle from rat while it remained unchanged in the more hypoxia-tolerant cardiac muscle from guinea pig and frog (Joseph et al., 2000). Decreases in the efficiency of the contractile system during hypoxia appear immediately easier to conceive than increases, and it should be stressed that there does not seem to exist any report on improved efficiency of cross bridge formation during hypoxia. Given the findings of the present study, it would be of interest to examine force development and rate of ATP hydrolysis in skinned myocardial tissue of turtle to evaluate cost of contraction directly.

Apart from the contractile system, differences in energy economy may reside in the excitation–contraction coupling. The excitation–contraction coupling in contracting cardiac muscle may account for a considerable fraction of energy consumption, which in mammals amounts to ~30% of energy turnover (Kammermeier, 1997). Furthermore, the relative importance of Ca^{2+} regulation by the sarcoplasmic reticulum and sarcolemmal Ca^{2+} transport varies substantially among ectothermic vertebrates (Tibbits et al., 1991; Driedzic and Gesser, 1994). Thus, possible differences in the excitation–contraction coupling between turtle and trout myocardium may influence the economy of the contracting myocardium.

Our finding of a decreased apparent cost of contraction expressed as increased ratio of force development to ATP production in the turtle cardiac muscle during hypoxia deviates from previous studies on perfused hearts of turtle and trout in which work output relative to ATP formation was similar under severe hypoxia and full oxygenation (Reeves, 1963b; Arthur et al., 1992, 1997). An explanation of the differences found between previous studies as well as between turtle and trout pertains to the assumptions used in the assessment of ATP production. Thus, comparing ATP regeneration during full oxygenation and severe hypoxia involves a risk that oxidative

ATP formation is overestimated in fully oxygenated preparations, as a proton leak across the inner mitochondrial membrane has been reported to account for a substantial fraction of oxygen consumption in isolated trout atrial myocytes (Mortensen and Gesser, 1999). This fraction is likely to be lowered during severe hypoxia, as indicated by mitochondrial experiments (Gnaiger et al., 2000). The possibility therefore exists that an overestimation of ATP production during oxygenation would exaggerate the apparent improvement of economy of contraction observed for turtle during hypoxia. By a similar argument, the economy of contraction would not stay unchanged but would be reduced for trout during hypoxia. While a comparison of turtle and trout would be obscured by a difference in mitochondrial proton leak, no evidence for such a species-related difference appeared, as turtle and trout preparations had similar force to ATP ratios during oxygenation. Thus, the economy of contraction during hypoxia differs between turtle and trout, and it seems likely that the ratio between force and ATP production does, indeed, increase in turtle.

Due to differences in living and acclimation temperatures, the turtle and trout experiments were carried out at 20 and 15°C, respectively. In a previous study on isolated trout cardiac muscle, twitch force and energy state were found to attain similar values after 60 min of anoxia at 10 and 20°C (Hartmund and Gesser, 1992). Thus, the qualitative differences between the two preparations should remain, although the 5°C difference in experimental temperature may have small quantitative effects.

In conclusion, this study raises the interesting possibility that turtle cardiac muscle relative to cardiac muscle of other species protects both the cellular energy state and force development during severe hypoxia in an energetically more efficient way. It is unclear how this relates to a downregulation of non-contractile energy demand, as the decrease in resting energy production was, somewhat surprisingly, about the same for trout as for turtle myocardium. However, a superior energy economy and energetic state may contribute to the ability of turtle cardiac muscle to maintain mechanical activity over long periods of severe hypoxia/anoxia.

This study was supported by the Danish Research Council. We thank Tobias Wang for thoughtful comments and Dorte Olsson for valuable technical assistance.

References

Allen, D. G., Morris, P. G., Orchard, C. H. and Pirolo, J. S. (1985). A nuclear magnetic resonance study of metabolism in the ferret heart during hypoxia and inhibition of glycolysis. *J. Physiol.* **361**, 185-204.

Altimiras, J. and Larsen, E. (2000). Non-invasive recording of heart rate and ventilation rate in rainbow trout during rest and swimming. Fish go wireless! *J. Fish Biol.* **57**, 197-209.

Arthur, P. G., Keen, J. E., Hochachka, P. W. and Farrell, A. P. (1992). The metabolic state of the *in situ* perfused trout heart during severe hypoxia. *Am. J. Physiol.* **263**, R798-R804.

Arthur, P. G., Franklin, C. E., Cousins, K. L., Thorarensen, H., Hochachka, P. W. and Farrell, A. P. (1997). Energy turnover in the normoxic and anoxic turtle heart. *Comp. Biochem. Physiol. A* **117**, 121-126.

Belkin, D. A. (1968). Anaerobic brain function: effects of stagnant and anoxic anoxia on persistence of breathing in reptiles. *Science* **162**, 1017-1018.

Bing, O. H. L., Brooks, W. W., Inamdar, A. N. and Messer, J. V. (1972). Tolerance of isolated heart muscle to hypoxia – turtle *vs* rat. *Am. J. Physiol.* **223**, 1481-1485.

Bøtker, H. E., Helligsø, P., Kimose, H. H., Thomassen, A. R. and Nielsen, T. T. (1994). Determination of high energy phosphates and glycogen in cardiac and skeletal muscle biopsies, with special reference to influence of biopsy technique and delayed freezing. *Cardiovasc. Res.* **28**, 524-527.

Christensen, M., Hartmund, T. and Gesser, H. (1994). Creatine kinase, energy-rich phosphates and energy metabolism in heart muscle of different vertebrates. *J. Comp. Physiol. B* **164**, 118-123.

Driedzic, W. R. and Gesser, H. (1994). Energy-metabolism and contractility in ectothermic vertebrate hearts – hypoxia, acidosis, and low temperature. *Physiol. Rev.* **74**, 221-258.

Farrell, A. P., Franklin, C. E., Arthur, P. G., Thorarensen, H. and Cousins, K. L. (1994). Mechanical performance of an *in situ* perfused heart from the turtle *Chrysemys scripta* during normoxia and anoxia at 5°C and 15°C. *J. Exp. Biol.* **191**, 207-229.

Ferguson, S. J. (1987). The ups and downs of P/O ratios (and the question of nonintegral coupling stoichiometries for oxidative phosphorylation and related processes). *Trends Biochem. Sci.* **11**, 351-353.

Gnaiger, E., Mendez, G. and Hand, S. C. (2000). High phosphorylation efficiency and depression of uncoupled respiration in mitochondria under hypoxia. *Proc. Natl. Acad. Sci. USA* **97**, 11080-11085.

Godt, R. E. and Nosek, T. M. (1989). Changes of intracellular milieu with fatigue or hypoxia depress contraction of skinned rabbit skeletal and cardiac muscle. *J. Physiol.* **412**, 155-180.

Hansen, J., Gesser, H. and Altimiras, J. (2002). Mechanical efficiency of the trout heart during volume and pressure-loading: Metabolic implications of the stiffness of the ventricular tissue. *J. Comp. Physiol. B* **172**, 477-484.

Hartmund, T. and Gesser, H. (1992). Temperature, contractility and high-energy phosphates in anoxic fish heart-muscle. *J. Comp. Physiol. B* **162**, 714-721.

Hartmund, T. and Gesser, H. (1996). Cardiac force and high-energy phosphates under metabolic inhibition in four ectothermic vertebrates. *Am. J. Physiol.* **271**, R946-R954.

Harwood, C. L., Young, I. S. and Altringham, J. D. (2002). How the efficiency of rainbow trout (*Oncorhynchus mykiss*) ventricular muscle changes with cycle frequency. *J. Exp. Biol.* **205**, 697-706.

Hicks, J. M. and Farrell, A. P. (2000). The cardiovascular responses of the red-eared slider (*Trachemys scripta*) acclimated to either 22 or 5°C. I. Effects of anoxic exposure on *in vivo* cardiac performance. *J. Exp. Biol.* **203**, 3765-3774.

Hicks, J. W. and Wang, T. (1998). Cardiovascular regulation during anoxia in the turtle: an *in vivo* study. *Physiol. Zool.* **71**, 1-14.

Hochachka, P. W., Buck, L. T., Doll, C. J. and Land, S. C. (1996). Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proc. Natl. Acad. Sci. USA* **93**, 9493-9498.

Jackson, D. C. (1968). Metabolic depression and oxygen depletion in diving turtle. *J. Appl. Physiol.* **24**, 503-509.

Jackson, D. C. (1987). Cardiovascular function in turtles during anoxia and acidosis: *in vivo* and *in vitro* studies. *Am. Zool.* **27**, 49-58.

Jackson, D. C. (2000). Living without oxygen: lessons from the freshwater turtle. *Comp. Biochem. Physiol. A* **125**, 299-315.

Jackson, D. C. (2002). Hibernating without oxygen: the painted turtle. *J. Physiol.* **543**, 731-737.

Jackson, D. C., Shi, H., Singer, J. H., Hamm, P. H. and Lawler, R. G. (1995). Effects of input pressure on *in vitro* turtle heart during anoxia and acidosis: a 31P-NMR study. *Am. J. Physiol.* **37**, R683-R689.

Jensen, M. and Gesser, H. (1999). Influence of inorganic phosphate and energy state on force in skinned cardiac muscle from freshwater turtle and rainbow trout. *J. Comp. Physiol. B* **169**, 439-444.

Joseph, T., Coirault, C. and Lecarpentier, Y. (2000). Species-dependent changes in mechano-energetics of isolated cardiac muscle during hypoxia. *Basic Res. Cardiol.* **95**, 378-384.

Julio, A. E., Montpetit, C. J. and Perry, S. F. (1998). Does blood acid–base status modulate catecholamine secretion in the rainbow trout (*Oncorhynchus mykiss*)? *J. Exp. Biol.* **201**, 3085-3095.

Kalinin, A. and Gesser, H. (2002). Oxygen consumption and force development in turtle and trout cardiac muscle during acidosis and high extracellular potassium. *J. Comp. Physiol. B* **172**, 145-151.

Kammermeier, H. (1997). Myocardial cell energetics. In *Advances in*

Experimental Medicine and Biology, vol. 430, *Analytical and Quantitative Cardiology* (ed. S. Sideman and R. Beyar), pp. 89-96. New York: Plenum Press.

Keiver, K. M., Weinberg, J. and Hochachka, P. W. (1992). Roles of catecholamines and corticosterone during anoxia and recovery at 5°C in turtle. *Am. J. Physiol.* **263**, R770-R774.

Lowry, O. H. and Passonneau, J. V. (1972). *A Flexible System of Enzymatic Analysis*. London: Academic Press.

Lutz, P. L. and Nilsson, G. E. (1997). Contrasting strategies for anoxic brain survival – glycolysis up or down. *J. Exp. Biol.* **200**, 411-419.

Mast, F. and Elzinga, G. (1990). Oxidative and glycolytic ATP formation of rabbit papillary muscle in oxygen and nitrogen. *Am. J. Physiol.* **258**, H1144-H1150.

Meyer, R. A. (1988). A linear model of muscle respiration explains monoexponential phosphocreatine changes. *Am. J. Physiol.* **254**, C548-C553.

Mortensen, B. and Gesser, H. (1999). O_2 consumption and metabolic activities in resting cardiac myocytes from rainbow trout. *J. Exp. Zool.* **283**, 501-509.

Nielsen, J. S. and Gesser, H. (2001). Effects of high extracellular [K^+] and adrenaline on force development, relaxation and membrane potential in cardiac muscle from freshwater turtle and rainbow trout. *J. Exp. Biol.* **204**, 261-268.

Nicol, S. C., Glass, M. L. and Heisler, N. (1983). Comparison of directly determined and calculated plasma bicarbonate concentration in the turtle *Chrysemys picta belli* at different temperatures. *J. Exp. Biol.* **107**, 521-525.

Reeves, R. B. (1963a). Control of glycogen utilization and glucose uptake in anaerobic turtle heart. *Am. J. Physiol.* **205**, 23-29.

Reeves, R. B. (1963b). Energy cost of work in aerobic and anaerobic turtle heart muscle. *Am. J. Physiol.* **205**, 17-22.

Reid, S. G. and Perry, S. F. (2003). Peripheral O_2 chemoreceptors mediate humoral catecholamine secretion from fish chromaffin cells. *Am. J. Physiol.* **284**, R990-R999.

Rolfe, D. F. S. and Brand, M. D. (1997). The physiological significance of mitochondrial proton leak in animal cells and tissues. *Biosci. Rep.* **17**, 9-16.

Shi, H. and Jackson, D. C. (1997). Effects of anoxia, acidosis and temperature on the contractile properties of turtle cardiac muscle strips. *J. Exp. Biol.* **200**, 1965-1973.

Syme, D. A. (1994). The efficiency of frog ventricular muscle. *J. Exp. Biol.* **197**, 143-164.

Tibbits, G. F., Hove-Madsen, L. and Bers, D. M. (1991). Calcium-transport and the regulation of cardiac contractility in teleosts – a comparison with higher vertebrates. *Can. J. Zool.* **69**, 2014-2019.

Wasser, J. S., Inman, K. C., Arendt, E. A., Lawler, R. G. and Jackson, D. C. (1990). 31P-NMR measurements of pH_i and high-energy phosphates in isolated turtle hearts during anoxia and acidosis. *Am. J. Physiol.* **28**, R521-R530.

Wasser, J. S. and Jackson, D. C. (1991). Effects of anoxia and graded acidosis on the levels of circulating catecholamines in turtles. *Resp. Physiol.* **84**, 363-377.

White, F. N. and Ross, G. (1966). Circulatory changes during experimental diving in the turtle. *Am. J. Physiol.* **211**, 15-18.